REMARKS

Applicants, through the undersigned, wish to thank Examiner Ton for the courtesy and assistance extended on behalf of Applicants during a personal interview conducted on February 28, 2006.

In the Final Action dated November 21, 2005, claims 39-46, 51, 56-58, 60-68, 86 and 88-89 are pending and are rejected.

This Response addresses each of the Examiner's rejections. Applicants therefore respectfully submit that the present application is in condition for allowance. Favorable consideration of all pending claims is therefore respectfully requested.

In the first instance, Applicants have amended the specification on page 76 to correct a typographical error. The reference to "FGF" on page 76, line 22, should be "bFGF" instead. The term "FGF" on page 76, line 22, is the only (incorrect) reference to FGF, instead of bFGF. Support for the amendment to the specification is found throughout the specification, including page 40, line 30 to page 41, line 2; page 41, line 29; and page 64, line 21. In light of the disclosure in the specification, particularly on page 64, line 21, those skilled in the art would understand that the reference to "FGF" on page 76, line 22 is a typographical error and should be "bFGF". No new matter is introduced.

Applicants have also amended the claims to more clearly delineate preferred embodiments of the present invention. Specifically, as presently recited, claims 39, 44-46, 94, and 100-101 are directed to methods of producing neural progenitor cells from hES cells; claims 64, 67-68, 94 and 102-104 are directed to methods of producing an enriched preparation of

neural progenitor cells from hES cells; and claims 51, 56-58, 60-61, 63 and 86 are directed to methods of inducing somatic differentiation of neural progenitor cells.

Independent claims 39 and 64 have been amended to further delineate the conditions for producing neural progenitor cells from hES cells, i.e., the cells are cultured "in the presence of serum free medium supplemented with growth factors which include epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF)". Support for these recited conditions is found in the specification, e.g., at page 76, lines 27-29 and page 77,lines 10-13. The neural progenitor cells produced are further characterized in the claims by the capacity to further differentiate "into neurons, into oligodendrocytes, and into astrocytes". This characterization is supported by the specification at pages 81-83, and delineates the multipotent capacity of the instant neural progenitor cells to differentiate into all three cell types. In light of the specification, those skilled in the art would understand, however, that the instant neural progenitor cells may differentiate into only one cell type given certain differentiation conditions. Furthermore, the neural progenitor cells are characterized in the claims by the combination of markers: polysialated N-CAM, nestin, vimentin and the transcription factor Pax-6. Support for this characterization is also found in the specification, e.g., page 79, lines 10-15.

Claims 51 and 56 have been amended to define the neural progenitor cells employed in the claimed methods by the differentiation capacity and by a combination of expression markers, as supported by the specification identified above.

Claims 60-61 have been amended to delineate two of the processes for inducing somatic differentiation of neural progenitor cells. Support for amended claims 60-61 is found in previous claims 60-62 and in the specification, e.g., in the bridging paragraph of pages 70-71.

New claims 100-104 depend from either claim 39 or claim 64, and further delineate conditions for culturing hES cells, prior to culturing in the presence of serum free media supplemented with growth factors. Support for these claims is found in claim 39, and in the specification on pages 35-37, for example.

No new matter is introduced by the foregoing amendments.

In the Office Action, claims 39-46, 51, 56-58, 60-68 and 86-99 are rejected under 35 U.S.C. §112, first paragraph, as failing to comply with the written description requirement.

The Examiner contends that there is insufficient description in the specification for the identification and generation of the neural progenitor cells (NPCs). The Examiner also states that the disclosure and figures, which Applicants previously identified as relevant descriptions, merely show that the cells produced by the claimed methods express certain markers. The Examiner states that the markers recited in the claims are also found in cell types other than neural progenitor cells. Regarding the transplantation data, the Examiner contends that the data merely demonstrate that the NPCs are capable of differentiation to various neural cell types, but the data themselves are not sufficient description for the NPCs.

Applicants respectfully submit that independent claims 39, 51, 56, 60-61 and 64, as amended, all define the neural progenitor cells by the multipotent differentiation capacity (i.e., to give rise to neurons, oligodendrocytes and astrocytes) and by a combination of expression markers. Independent claims 39 and 64 also define specific conditions for producing the neural progenitor cells from hES cells.

Applicants respectfully submit that the specification has demonstrated consistent generation of neural progenitor cells, identified by the recited combination of expression markers

and the multipotent differentiation capacity. See pages 79 and 81-83 of the specification, for example. Therefore, Applicants respectfully submit that the specification has clearly described the neural progenitor cells, as presently recited, in a manner that conveys to those skilled in the art that the inventors were in possession of the neural progenitor cells at the time the present application was filed. Applicants respectfully submit that the neural progenitor cells, as presently recited, are adequately described in the specification in full compliance with the written description requirement under 35 U.S.C. §112, first paragraph. Withdrawal of the rejection is respectfully requested.

Claims 39-46, 51, 56-58, 60-68, 86, 88-99 are rejected under 35 U.S.C. §112, first paragraph, as failing to comply with the enablement requirement.

The Examiner contends that the claims broadly recite "a controlled differentiation condition", which would take undue experimentation to determine based on the state of the art at the relevant time. In addition, the Examiner contends that because of a lack of description for NPCs, those skilled in the art would not be able to determine whether the cells produced by the claimed methods are NPCs or not.

In the first instance, Applicants respectfully submit that the claims have been amended to define the neural progenitor cells by a combination of expression markers and a multipotent differentiation capacity. These amendments are believed to have adequately addressed the Examiner's concern regarding insufficient description of the neural progenitor cells.

Furthermore, Applicants respectfully submit that independent claims 39 and 64 have been amended to recite specific culture conditions for producing and enriching neural progenitor cells. Applicants respectfully submit that the specification provides ample guidance for

practicing the methods of producing neural progenitor cells from hES cultures, as presently amended. Applicants direct the Examiner's attention to pages 39-41 of the specification, where the claimed methods are described generally, to pages 64-66, where the specification provides details of the methods, and to pages 76-77, where specific exemplification are provided. The specification further identifies the importance of serum free media, preferably supplemented with growth factors, in supporting the proliferation of cells of the neural lineage and eliminating other unwanted differentiated cell types during culturing, thereby enriching neural progenitor cells. See, e.g., page 40, line 25-page 41, line 5, page 41, lines 10-13, page 41, line 29 to page 42, line 4.

Moreover, Applicants respectfully submit that independent claims 51, 56, and 60-61, also delineate specific conditions for inducing further differentiation of neural progenitor cells. The specification also provides clear guidance, including specific examples, for these claimed methods. See, e.g., pages 69-72 and 81-83.

Accordingly, Applicants respectfully submit the specification provides ample guidance for those skilled in the art to practice the claimed methods, i.e., to produce NPCs from hES cells in the presence of serum free media and growth factors, to further propagate these cells as NPCs provided that the growth factors are present, and to further differentiate the NPCs to, for example, oligodendrocytes, astrocytes and neuronal cells, by withdrawal of the growth factors. Applicants submit that based on the present teaching, it would not take undue experimentation to practice the methods as presently claimed. Therefore, withdrawal of the enablement rejection is respectfully requested.

Claims 39-46 are rejected under 35 U.S.C. §102(b) as anticipated by Shamblott et al. (1998).

Applicants observe that in Shamblott, pluripotent germ cells (PGS) from gonadal ridges and mesenteries were cultured to produce pluripotent embryonic germ (EG) cells. The EG cells were cultured to generate embryoid bodies (EBs), which were shown to contain various cell types, including ectoderm derivatives of cells suggestive of neuroepithelial and anti-neurofilament-reactive cells.

In the first instance, Applicants respectfully submit that EG cells and hES cells are distinct cell types despite their pluripotentiality. There are epigenetic differences between the two cell types reflecting a somatic (ES) versus a germline (EG) origin. These differences in so-called paternal/maternal imprinting are well recognized in the art, as reviewed in Zwaka and Thomson (*Development* 132: 227-233, 2005, copy enclosed) (see page 229).

Furthermore, the present methods, as characterized in independent claim 39, employ serum free media supplemented with growth factors to produce NPCs. Shamblott does not teach anywhere the use of serum free media to produce NPCs.

Moreover, the presently claimed methods produce NPCs that are characterized by a combination of expression markers and a multipotent differentiation capacity. Shamblott does not teach anywhere production of neural progenitor cells as presently characterized.

Accordingly, Shamblott does not teach each and every element of the claimed invention. Withdrawal of the §102(b) rejection based on Shamblott is respectfully requested.

Claims 51, 95 and 99 are rejected under 35 U.S.C. §103(a) as allegedly unpatentable over Thomson et al. (Science 282: 1145-1147, 1998) in view of Brustle et al. (Science 285: 754-756, 1999). The remaining claims are rejected as allegedly unpatentable over Thomson et al. in

view of Brustle et al., and further in view of either or both of Stemple et al. (Cell 71: 973-985, 1992) and Ben-Hur et al. (J. Neurosci. 18: 5777-5788, 1998).

Claims 95 and 99 have been canceled, rendering the rejection of these claims moot. However, as claims 39 and 64, and their dependent claims now recite culturing cells in the presence of serum free media and growth factors, Applicants will address the §103 rejection to the extent the rejection may be applied by the Examiner to these claims as well.

The Examiner contends that Thomson teaches hES cells, and that Brustle teaches inducing differentiation of mES cells to glial precursors in the presence of DMEM/F12 media supplemented with FGF2 and PDGF-AA and on polyornithine-coated dishes. Brustle further teaches that withdrawal of the growth factors induce the progenitor cells to differentiate into oligodendrocytes. The Examiner concludes that it would have been obvious for one skilled in the art to culture the hES cells of Thomson, under the conditions taught by Brustle, to arrive at the claimed invention, with a reasonable expectation of success.

In the first instance, Thomson teaches differentiation of hES cells *in vitro* into trophoblast and endoderm lineages. Neither of these lineages is relevant to neural cell types, which derive from the ectoderm layer. Thomson also demonstrates the broader developmental potential of hES cells into derivatives of all three germ layers; however, this is only shown *in vivo* in the teratoma example. Given the contrast in the Thomson reference between the *in vivo* (teratoma) and *in vitro* differentiation results, those skilled in the art would conclude that it would be difficult to direct differentiation of hES cells towards the neural lineage *in vitro*. Therefore, Applicants respectfully submit that Thomson does not provide any motivation for those skilled in the art to culture hES cells *in vitro* to generate neural progenitor cells that could be further differentiated into neuronal cells, into oligodendrocytes or into astrocytes.

With respect to Brustle, Applicants respectfully submit that Brustle's teaching is entirely directed to culturing mouse ES cells. Applicants respectfully submit that it was evident at the time the priority case of the present application was filed in 2000, that the differences between mES cells and hES cells were so substantial that those skilled in the art would not have had a reasonable expectation that hES cells would behave in the same manner as mES cells. Therefore, those skilled in the art would not have had a reasonable expectation of success in producing human NPCs and to further differentiate such human NPCs by simply applying the conditions developed using mES cells, taught by Brustle, to human ES cells.

In support of Applicants' position, Applicants are providing herewith a Declaration of Dr. Alan Colman under 37 C.F.R. §132. As stated in the Declaration, it is Dr. Colman's opinion that the differences between hES cells and mES cells were known to be significant in 2000 such that those skilled in the art would not be able to readily extrapolate the results achieved with mES cells to hES cells. Dr. Colman concludes that it is his opinion, at the time the priority document of the present application was filed, those skilled in the art would not have had any reasonable expectation of success if they were to apply the differentiation conditions developed with mES cells, as taught by Brustle, to hES cells in order to obtain human neural progenitor cells.

The Examiner argues that the differences between human and mouse ES cells reside in the conditions for maintaining the cells, not for differentiating the cells. Applicants respectfully submit that these conditions are inextricably linked. The manner in which the cells are maintained in culture will vastly affect the outcome upon induction of differentiation, as described below, and the manner in which mES cells are cultured and behave are vastly different from hES cells.

Further with respect to Brustle, Applicants respectfully submit that this reference teaches the derivation of glial precursors which can differentiate to oligodendrocytes and astrocytes only. In Brustle, no neurons were formed from the precursor cells, regardless of whether the precursors were cultured *in vitro* in the absence of growth factors or transplanted into rat spinal cords *in vivo*. Applicants respectfully submit that the methods, as presently recited, are directed to the production or further differentiation of neural progenitor cells that can form all three different cell types, namely a neuronal cell, oligodendrocyte and an astrocyte. Applicants respectfully submit that in contrast to the claimed methods, Brustle does not teach or suggest how to prepare multipotent neural progenitor cells, as presently characterized, much less how to further differentiate such cells into cells such as neuronal cells. Therefore, those skilled in the art not only would not have had a reasonable expectation of success in applying the conditions for culturing mouse ES cells, taught by Brustle, to human ES cells, but also would not have had a reasonable expectation of success in applying the conditions taught by Brustle to produce the multipotent human neural progenitor cells as presently claimed.

In view of the foregoing, Applicants respectfully submit that neither Thomson nor Brustle provide motivation to combine the teachings of the cited references to apply the conditions taught by Brustle to human ES cells in order to produce human NPCs. Further, even assuming, *pro arguendo*, some motivation was provided, those skilled in the art would not have had a reasonable expectation of success in applying the conditions taught by Brustle to human ES cells to produce human NPCs, much less human NPCs having the multipotent differentiation capacity, as presently claimed.

Therefore, the obviousness rejection based on the combination of Thomson and Brustle is overcome. Withdrawal of the rejection is respectfully requested.

In view of the foregoing amendments and remarks, it is firmly believed that the subject application is in condition for allowance, which action is earnestly solicited.

Respectfully submitted,

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Encls.:

- Zwaka and Thomson (2005)
- Declaration of Alan Colman (with attached Exhibits A-H)

A germ cell origin of embryonic stem cells?

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Summary

Because embryonic stem (ES) cells are generally derived by the culture of inner cell mass (ICM) cells, they are often assumed to be the equivalent of ICM cells. However, various evidence indicates that ICM cells transition to a different cell type during ES-cell derivation. Historically, ES cells have been believed to most closely resemble pluripotent primitive ectoderm cells derived directly from the ICM. However, differences between ES cells and primitive ectoderm cells have caused developmental biologists to question whether ES cells really have an in vivo equivalent, or whether their properties merely reflect their tissue culture environment. Here, we review recent evidence that the closest in vivo equivalent of an ES cell is an early germ cell.

Introduction

Embryonic stem (ES) cells are pluripotent (see Box 1) and can be expanded without limit in vitro (Evans and Kaufman, 1981; Martin, 1981; Thomson et al., 1998). It is remarkable that permanent pluripotent stem cell lines can be derived from pre-implantation embryos at all, because, in vivo, pluripotent cells of the early mammalian embryo proliferate only briefly before becoming cells with a more restricted developmental potential. A few years after the initial derivation of mouse ES cells, it was suggested that they be called 'embryo-derived stem cells', a more precise term that would distinguish between these new pluripotent cell lines and cells within the embryo (Rossant and Papaioannou, 1984). However, this term was never adopted, and the extent to which these pluripotent stem cell lines represent any specific embryonic cell type or reflect their artificial tissue culture environment is still an open issue today – two decades later. Elucidating the origin of ES cells is of importance because it may help us to identify genes that are essential for the long-term maintenance of the pluripotent state. It could also assist with the derivation of ES cells from species whose ES cells have proved difficult to isolate. It will also help us to assess how accurately ES cell differentiation reflects events that normally occur in vivo. Here, we review the origin of ES cells, and explore recent evidence that ES cells are closely related to early germ cells.

The historical origins of ES cells: embryonal carcinoma cells

Historically, work with mouse teratocarcinomas paved the way for the derivation of ES cells. These germ cell tumors contain multiple differentiated tissues and undifferentiated stem cells, called embryonal carcinoma (EC) cells (Damjanov and Solter, 1974; Dixon and Moore, 1952; Kleinsmith and Pierce, 1964). Although teratocarcinomas had been known as medical curiosities for centuries (Wheeler, 1983), it was the discovery that male mice of strain 129 had a high incidence of testicular teratocarcinomas (Stevens and Little, 1954) that made these tumors more routinely amenable to experimental analysis. Because their growth is sustained by the persistent EC cell component (Stevens and Little, 1954), teratocarcinomas can be serially transplanted between mice. Eventually, conditions were developed that allowed the culture of EC cells in vitro, establishing them as an in vitro model of mammalian development (Kahan and Ephrussi, 1970).

As pluripotent cells of the intact early embryo proliferate for only a limited period of time, it was not initially obvious whether pluripotent cell lines could be established without undergoing malignant transformation. However, the transplantation of genital ridges or of egg-cylinder-stage embryos into ectopic sites, such as under the kidney capsule of adult mice, gave rise to teratocarcinomas at a high frequency in strains that did not spontaneously produce these tumors (Solter et al., 1970; Stevens, 1970a; Stevens, 1970b). These teratocarcinomas could be serially transplanted between adult mice, depending on whether the EC cell component persisted or differentiated (Solter et al., 1981). If the EC compartment disappears, the resulting tumor develops as a benign teratoma. Indeed, the malignant phenotype of EC cells often depends on the strain of the host mouse, and not on the tumor strain. EC cells injected into mouse blastocysts can contribute to either the normal tissues of the resulting chimera (Brinster, 1974) or, in some cases, to tumors (Rossant and McBurney, 1982). Because the ectopic transplantation of normal peri-implantation embryos can give rise to pluripotent cell lines, the direct derivation of pluripotent cell lines in vitro was attempted without the teratocarcinoma step. The culture conditions that were established to support mouse EC cells, including the use of feeder cell layers, were essentially those used to isolate mouse, and eventually human, ES cells (Evans and Kaufman, 1981; Martin, 1981; Thomson et al., 1998).

One indication that these early EC cell lines may be derived from germ cells (Solter et al., 1970; Stevens, 1967; Stevens, 1970a) came from mouse genital ridge-transplantation experiments. These experiments showed that genital ridges

Box 1. Glossary

Inner cell mass (ICM)
The second lineage of the early embryo that is located inside the blastocyst. It gives rise to all embryonic tissues.

Pluripotency
Refers to the unique ability of cells within the early embryo to differentiate into all cell types.

Primitive ectoderm
The remaining ICM tissue formed during the second differentiation event of embryonic development (also known as epiblast or embryonic ectoderm)

Primitive endoderm
An epithelial layer derived from cells that are in contact with the blastocyst cavity.

Trophectoderm
During the first differentiation event in mammalian development, morula cells segregate into two cell lineages: the first, the trophectoderm, forms the outer layer of the blastocyst. It eventually becomes part of the placenta.

effectively give rise to teratocarcinomas only in a narrow time window (E12.0-12.5). It is around this time that migratory germ cells start arriving in the genital ridge. In the egg cylinder-transplantation experiments, however, the origin of the EC cells was less clear. Primitive ectoderm seemed the most likely candidate for several reasons: because the potential to form teratocarcinomas is lost at the time when primitive ectoderm disappears at E8.5 (Damjanov et al., 1971); because EC cells have phenotypic similarities to primitive ectoderm cells in vivo (Diwan and Stevens, 1976); and because EC cells, when reintroduced into blastocysts, contribute to the same tissues as primitive ectoderm (Brinster, 1974). In addition, when analyzing the earliest stages of teratocarcinoma formation in 129/Sv mouse fetal gonads, Stevens observed clusters of polarized epithelial cells surrounding a central cavity that morphologically resembled primitive ectoderm cells (Stevens, 1983). He also observed that the formation of teratocarcinomas in ovaries included parthenogenic activation of the oocyte, the formation of blastocyst-like structures and the subsequent formation of structures that resembled early egg cylinders, which eventually became disorganized. Isolated transplanted primitive ectoderm itself gives rise to teratocarcinomas (Diwan and Stevens, 1976), but because early germ cells are just appearing at this stage, a germ cell origin cannot be completely ruled out by these experiments.

Are ES cells a tissue culture artifact?

ES cells clearly exhibit some properties that are not normally shown by cells of the intact embryo. For example, although ES cells retain properties of early embryonic cells in vitro, no pluripotent cell demonstrates long-term self-renewal in vivo. Embryonic cells, once brought into tissue culture, are exposed to numerous extrinsic signals to which they never would be exposed to in vivo. ES cells certainly adapt to selective tissue culture conditions and acquire novel functions that allow them to proliferate in an undifferentiated state indefinitely, and, because of this, ES cells are in some sense tissue culture artifacts (Buehr and Smith, 2003; Rossant, 2001; Smith, 2001).

As these changes are inevitable, the issue is not whether ES cells exhibit some properties that merely reflect their tissue culture environment, but rather whether they are most closely related to a specific in vivo cell type in the embryo, or if the influence of the culture environment is so dominant that it is impossible to relate ES cells to a single, in vivo cell type. We will certainly not completely resolve this issue here, but will re-explore the relationship of ES cells to specific early embryonic cell types.

Are ES cells most closely related to primitive ectoderm?

Although ES cell lines are generally derived from the culture of the ICM, some experiments suggest that ES cells more closely resemble cells from the primitive ectoderm. For example, isolated primitive ectoderm from the mouse gives rise to ES cell lines at a higher frequency than does isolated ICM. Moreover, the culture of primitive ectoderm allows the isolation of ES cell lines from mouse strains that have been previously refractory to ES cell isolation (Brook and Gardner, 1997). Indeed, ES cell lines can be derived from single, isolated, mouse primitive ectoderm cells, which is not possible with ICM cells (Gardner and Brook, 1997). Although these experiments suggest that ES cells are more closely related to primitive ectoderm than to ICM, they do not reveal whether ES cells more closely resemble primitive ectoderm or a cell derived from it in vitro.

A maximum of three individual cultured primitive ectoderm cells per embryo have been shown to give rise to ES cell colonies (Gardner and Brook, 1997). This low frequency could have been due to some variability in the potential of primitive ectoderm cells, to some variability in the environment in which they were placed or to damage caused by the dissociation of the primitive ectoderm into individual cells. However, by tracking the expression of the octamer-binding transcription factor 4 (*Oct4*) gene, a marker of pluripotency, in intact cultured ICM/epiblast cells, it was shown that *Oct4* expression was maintained in only a small proportion of outgrowing cells (Buehr et al., 2003), which also suggests that only a minority of primitive ectoderm cells can transit to a new stable, proliferative pluripotent state, and, subsequently, be expanded as ES cells. These results could be due to a requirement for a relatively rare intrinsic or extrinsic stochastic event, or to an inherent heterogeneity of the primitive ectodermal cell population. Recent data indicate that even the earliest ICM is heterogeneous and consists of a mixture of cells that express either *Oct4* or *Gata6* (Rossant et al., 2003), and a similar later heterogeneity could account for the fact that only a minority of primitive ectoderm cells generally give rise to ES cells in culture.

Established mouse ES cell lines express some specific markers of primitive ectoderm at a very low level, if at all (Table 1), such as fibroblast growth factor 5 (*Fgf5*) (Haub and Goldfarb, 1991; Hebert et al., 1991; Rathjen et al., 1999). Culture conditions have been established that convert mouse ES cells into early primitive ectoderm-like cells that express both *Fgf5* and *Oct4* (Rathjen et al., 1999), but these cells fail to form chimeras when injected into mouse blastocysts. Taken together, these results suggest that ES cells are most closely

Table 1. Marker genes expressed in embryonic stem cell (ES), early germ (EGC) and later germ cells (LGC), in the inner cell mass (ICM) and in the primitive ectoderm (PE)*

Gene	Species	ES	EGC	LGC	ICM	PE
Pou5f1 (Pesce and Scholer, 2001)	M	+	+	+	+	+
Nanog (Chambers et al., 2003)	M	+	+	+	+	+
Dppa3 (Saitou et al., 2002)	M	+	+	+	+	+
Ifitm3 (Saitou et al., 2002)	M	+	+	+	+	+
Kit (Horie et al., 1991)	M	+	+	+	–	N/D
DAZL (Clark et al., 2004)	H	+	+	+	–	N/D
Ddx4 (Toyooka et al., 2003)	M	–	–	+	–	–
Akp2 (Chiquoine, 1954)	M	+	+	+	+	+
Zfp42 (Rogers et al., 1991)	M	+	N/D	N/D	+	–
Fgf5 (Haub and Goldfarb, 1991; Hebert et al., 1991)	M	–	N/D	N/D	–	+
Gbx1 (Chapman et al., 1997)	M	+	N/D	N/D	+	–

*Data are based on mouse (M) and human (H) studies, some are preliminary.
+ denotes expression at that developmental stage, – denotes the gene is not expressed
N/D, not done.

related to a subpopulation of primitive ectoderm cells, or to a close derivative of primitive ectoderm cells.

One of the curious species-specific differences between human and mouse ES cells is that human ES cells give rise to trophoblast cells at a high efficiency (Xu et al., 2002), but mouse ES cells do not (Beddington and Robertson, 1989). In the intact mouse embryo, the last cells capable of giving rise to trophoblast cells are early ICM cells, so the failure of mouse ES cells to differentiate into trophoblast is good evidence that they are not the equivalent of early ICM cells (Brook and Gardner, 1997). The differentiation of human ES cells to trophoblast could be explained if they are related to an earlier cell type than mouse ES cells, or if the specification of the trophoblast lineage occurs differently in human embryos. However, a third possibility is that ES cells represent a different cell type altogether. It is therefore worthwhile examining the relationship between ES cells and germ cells.

Germ cells and the primitive ectoderm

In elegant, clonal-fate mapping studies in the mouse (Lawson and Hage, 1994), germ cells were shown to arise from a founder population in the E6.0-6.5 proximal epiblast adjacent to the extra-embryonic ectoderm. These founder cells then pass through the primitive streak and give rise to several extra-embryonic mesodermal lineages and to germ cells. By E7.25, a distinct cluster of ~45 tissue non-specific, alkaline phosphatase (Tnap)-positive germ cells is present at the base of the allantois (Fig. 1) (Ginsburg et al., 1990). The E6.5 distal epiblast, which would not normally contribute to germ cells, will contribute to germ cells if transplanted to a proximal location (Tam and Zhou, 1996), which demonstrates that location and inductive signals, rather than germ plasm determinants, are responsible for the specification of germ cells in mice (Extavour and Akam, 2003). This flexibility suggests that cultured primitive ectoderm cells could spontaneously give rise to early germ cells in culture.

Bone morphogenetic protein 4 (Bmp4) (Lawson et al., 1999) and Bmp8b (Ying et al., 2000) are required for the formation of the proximal posterior extra-embryonic region that gives rise to primordial germ cells (PGCs) and to cells of the allantois in the mouse. The addition of Bmp4 and Bmp8b to distal mouse epiblast cultures increases the formation of cells strongly positive for Tnap (Ying et al., 2001), a marker shared by early germ cells and ES cells. These Tnap-positive cells were interpreted as being germ cells in this study. Recently, BMP signaling has been shown to be important for the self-renewal of mouse ES cells (Ying et al., 2003), and although BMPs are involved in many differentiation decisions in the early embryo, these results do further hint at a relationship between ES cells and early germ cells.

Similarities between germ cells and ES cells

In mice, PGCs migrate and proliferate until ~25,000 are present in the genital ridge at E13.0 (Tam and Snow, 1981). Pluripotent cell lines from pre- and post-migratory (Resnick et al., 1992; Matsui et al., 1992; Shamblott et al., 1998), as well as from migratory (Durcova-Hills et al., 2001), germ cells have been isolated, and these cell lines are termed embryonic germ (EG) cells to distinguish their origin. Mouse EG cell lines are remarkably similar to mouse ES cell lines (Donovan and de Miguel, 2003). During germ cell migration and maturation, however, the somatic status of imprinted genes is progressively erased (Yamazaki et al., 2003), and EG cells isolated at various stages of migration retain some of these differences, such as the reduced methylation of many imprinted genes, including *H19* and *Snrpn* (Hajkova et al., 2002). The analysis of mouse PGCs at E10.5 suggests that methylation erasure has already begun by this time, as supported by studies of the expression of imprinted genes (Yamazaki et al., 2003). This study showed that imprinted genes, such as *H19* and *Snrpn*, exhibit imprinted (somatic) expression patterns in E9.5 PGCs, but by E10.5 have switched to a bi-allelic mode of expression (Yamazaki et al., 2003). Because the genes expressed in ES cells exhibit somatic imprinting patterns (Geijsen et al., 2004), their change in imprinting status suggests that if ES cells are derived from germ cells, this derivation must occur before E9.5.

There is a paucity of known molecular markers that distinguish early germ cells from other pluripotent cells of the early embryo. One marker, Tnap, is strongly expressed by early germ cells and by ES cells, but is weakly expressed by the epiblast and other surrounding embryonic cells (Chiquoine, 1954; Ginsburg et al., 1990). Two new markers for early germ cells, fragilis (Ifitm3 – Mouse Genome Informatics) and Dppa3 (also know as stella or PGC7), have recently been identified that allow the better separation of early germ cell precursors from their differentiated neighboring cells (Saitou et al., 2002).

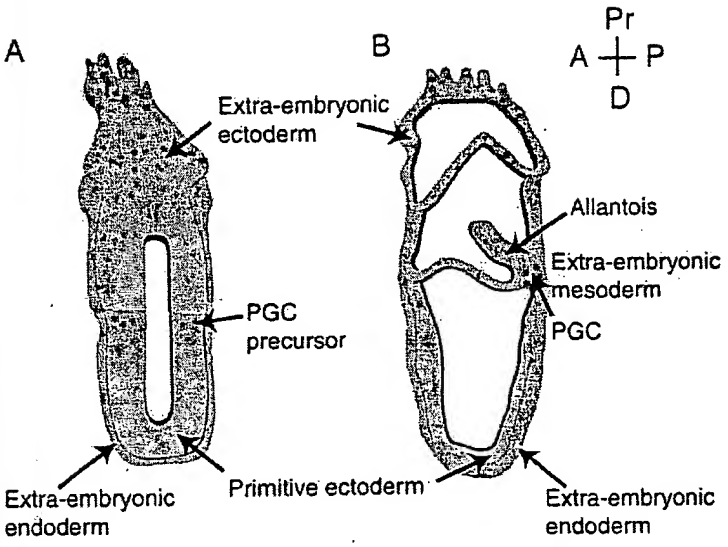

Fig. 1. Early development of the mouse embryo. (A) Six days after fertilization (E6.25), the mouse embryo consists of three layers. The inner cell mass (ICM) cells that are in contact with the blastocyst cavity differentiate into an epithelial layer called the extra-embryonic (primitive) endoderm. The rest of the ICM becomes the epiblast (primitive ectoderm). Primordial germ cells (PGCs, red dots) arise from a cell population in the proximal epiblast adjacent to the extra-embryonic ectoderm. These cells then pass through the primitive streak and give rise to several extra-embryonic mesodermal lineages and to germ cells. (B) By E7.25, a distinct cluster of ~45 tissue non-specific, alkaline phosphatase (Tnap)-positive PGCs is present at the base of the allantois within the extra-embryonic mesoderm (red dots). Once these PGCs are specified, they begin to migrate to the future gonadal anlagen. A, anterior; P, posterior; Pr, proximal; D, distal.

Dppa3 is expressed in pre-implantation embryos and in germ cells (Sato et al., 2002) and has recently been reported to have a role as a maternal transcript in preimplantation embryonic development (Bortvin et al., 2004). Dppa3-positive cells show increased expression of fragilis and remain positive for *Tnap* (*Akp2* – Mouse Genome Informatics) and *Oct4* (Saitou et al., 2002). Once Dppa3-positive PGCs start to migrate, they begin to express additional markers, such as steel factor receptor, followed by markers of more mature germ cells, such as murine vasa homolog (MVH; Ddx4 – Mouse Genome Informatics) (Saitou et al., 2002).

Several recent reports describing the differentiation of mouse ES cells into cells that express markers of mature male and female germ cells (Geijsen et al., 2004; Hubner et al., 2003; Toyooka et al., 2003) are important for our understanding of the origin of ES cells. In each of these reports, germ cell markers were expressed by ES cells themselves, including those, such as Dppa3, that help distinguish germ cells from primitive ectoderm (Table 1). Only the expression of more mature germ cell markers (such as MVH) enabled in vitro-derived germ cells to be distinguished from ES cells themselves. In one study that examined the differentiation of human ES cells into germ cells (Clark et al., 2004), the expression of each of eight genes that are characteristic of early germ cells was detected in human ES cells, but the expression of each of six genes that are characteristic of later germ cells was not detected, strongly suggesting that the expression of the early germ cell-genes was not merely a result of the broadly 'leaky' transcription that is often attributed to ES cells. Using immunocytochemistry, it was also shown that most individual human ES cells in a population express the early germ cell markers stella related (STELLAR) and deleted in azoospermia-like (DAZL), indicating that a minor subset of randomly differentiating cells in a mixed population is not responsible for the expression of germ cell markers in ES cell cultures. Importantly, it was also shown that at least one germ cell-specific gene, *DAZL*, was expressed by human ES cells but not by human ICM. The existing gene expression data, then, are consistent with the idea that the closest in vivo equivalent to ES cells is not the ICM or primitive ectoderm, but an early germ cell.

Some of the properties of ES cells, however, suggest that they are not merely the equivalent of early germ cells. For example, the earliest PGCs do not self-renew for prolonged periods of time, but instead begin a series of maturation steps, beginning with germ cell migration and ending in the highly specialized development of sperm or egg (Wylie, 1999). Although ES cells can differentiate into more mature germ cells in vitro, they do so relatively inefficiently. Indeed, the ability to colonize the germline of chimeras is one of the most easily lost properties of ES cells. If ES cells most closely represent early germ cells, it is unclear why they are not better at giving rise to more mature germ cells. In addition, isolated PGCs have never been demonstrated to contribute to chimeras when injected into blastocysts, so an exact equivalence to ES cells is unlikely.

Because a comprehensive and comparative analysis of the transcriptomes of isolated ICM, primitive ectoderm and early germ cells has not yet been reported, it is not yet clear how much the particular repertoire of genes expressed by ES cells represents an early germ cell, another specific in vivo cell type, a response to the tissue culture environment, or a combination of all three. If the ICM and primitive ectoderm are inherently heterogeneous, transcriptome analysis may need to be carried out at the single-cell level to ultimately understand these relationships. However, at the moment, the greatest concordance of known markers appears to be between ES cells and early germ cells.

Conclusions

We hypothesize that ES, EC and EG cells represent a family of related pluripotent cell lines, whose common properties reflect a common origin from germ cells (Fig. 2). Although a more detailed transcriptional analysis could ultimately refute the proposed relationship between ES cells and early germ cells, we hope this idea will at least help to stimulate a healthy

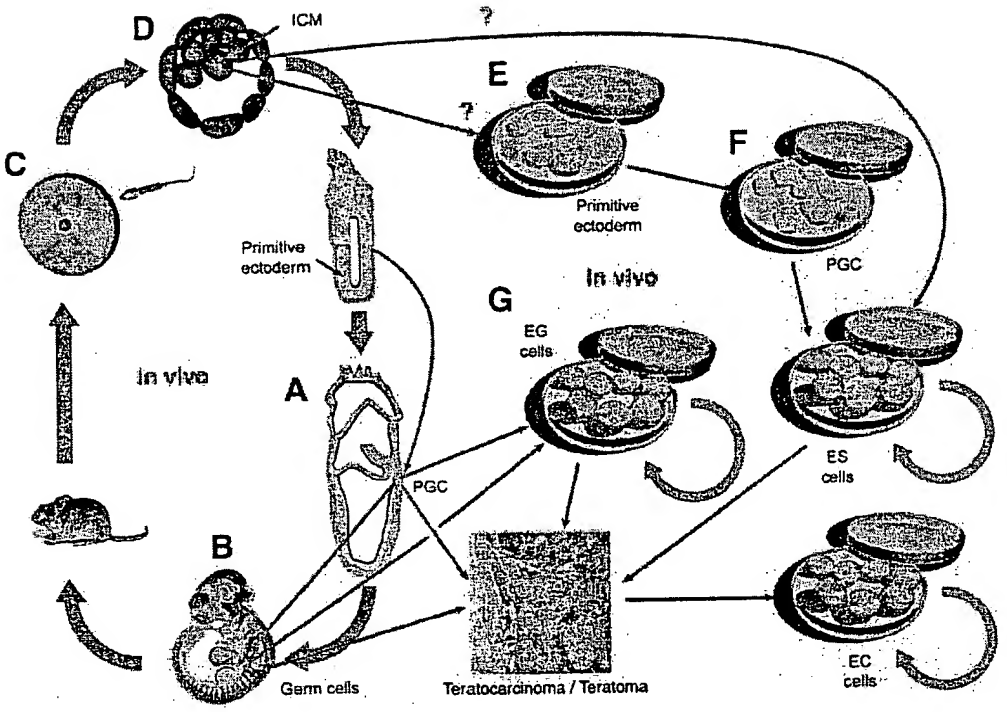

Fig. 2. Germ cell propagation in vitro and in vivo. (A-C) The germ cell cycle in the mouse. (A) Primordial germ cells (PGCs) appear at E7.25 as a small group of cells (red) in the extra-embryonic mesoderm. (B) After E8.5, PGCs start to migrate to the gonadal anlagen and contribute (C) during puberty to oocytes or sperm. (D) Embryonic stem (ES) cells are in vitro derivatives of inner cell mass (ICM) cells. (E,F) The formation of ES cells occurs either directly from the ICM/primitive ectoderm (E) or according to our hypothesis, through in vitro differentiation of ICM outgrowth into primitive ectoderm, then into extra-embryonic mesoderm and finally into PGCs (F). (G) PGCs that form in vivo (A-C) give rise to embryonic germ (EG) cells in vitro. Germ cells, PGCs, ES and EG cells are all capable of forming teratomas and teratocarcinomas. ES and EG cells can reintegrate into the normal embryo after injection into the blastocyst (not shown). Circular green arrows denote unlimited self-renewal.

re-evaluation of what is actually being studied when ES cells differentiate in vitro.

What is the relevance of a putative close relationship between ES cells and early germ cells? One prediction of this hypothesis is that at least some of the germ cell-specific genes expressed by ES cells, and not by primitive ectoderm cells, are essential for the long-term maintenance of the pluripotent state. If true, then it should be possible to generate knockout mice to identify genes that are essential for the specification or maintenance of PGCs, which are also essential for the derivation of ES cell. A related prediction of the hypothesis is that genes that are responsible for increasing susceptibility to spontaneous germ cell tumors should increase the efficiency of ES cell derivation. It is interesting, for example, that in species where teratocarcinomas occur at a clinically significant frequency, such as in mouse and human, ES cells have been successfully derived, whereas in species where teratocarcinomas are exceedingly rare, such as the rat, ES cells have proven difficult to derive. Understanding basic species differences in the specification or maintenance of early germ cells could allow the derivation of ES cells from species that have been hitherto resistant to the isolation of ES cells, such as the rat (Buehr et al., 2003).

Another implication of our hypothesis is that when looking for evolutionary clues to understand the pluripotent state, the comparative germ cell literature will be the most instructive. In a species such as the zebrafish, which has a germ plasm that strictly separates germ cells from somatic cells, it makes sense that pluripotent cell lines that can contribute to the germline in chimeras (Ma et al., 2001) would have to be derived from germ line-lineage cells.

Another prediction arising from the hypothesis that ES cells most closely represent early germ cells is that the very earliest events of ES cell differentiation into somatic and extra-embryonic lineages will not accurately reflect events that normally occur in vivo. The idea that ES cells represent an in vitro equivalent to the ICM, however, is firmly entrenched and continues to strongly influence our thinking about these cells. When examining the differentiation of ES cells in vitro, the pervasive mental image is of a forward progression that recapitulates normal embryonic events. For example, one thinks of ICM cells progressing to primitive ectoderm cells, then to neural ectoderm cells, and finally to more specialized neural cell types. If ES cells most closely represent early germ cells, this mental image needs revision, as the earliest transition would appear to be more 'lateral' or even 'backward' than 'forward'. It will be illuminating to define each of the distinct transitions that ES cells can make in a single step and to determine how much these initial transitions resemble in vivo or artificial differentiation. If ES cells really represent early germ cells, the initial events in differentiation would be expected to be transitions that do not normally occur in intact

embryos, except, perhaps, when the transition is to more mature germ cells.

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